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Antioxidant Compounds Isolated from the Roots of *Phlomis umbrosa* Turcz.

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Abstract – Two triterpenoids, arjunolic acid (**1**), belleric acid (**2**), five phenylethanoids, martynoside (**3**), orobanchoside (**4**), 3,4-dihydroxyphenethylalcohol-6-*O*-caffeoyl-*β*-D-glucoside (**5**), leucosceptoside B (**6**), lunarii-folioside (**7**), four phenolic acids, ferulic acid (**8**), syringic acid (**9**), vanillic acid (**10**), 4-hydroxybenzoic acid (**11**), and one lignan, (+)-syringaresinol-*β*-D-glucoside (**12**), were isolated from the roots of *P. umbrosa*. All isolated compounds were explored for their antioxidant potential in the DPPH and ABTS assays. In DPPH assay, compound **5** showed high antioxidant capacity. Compounds **3**, **4**, **6**, and **7** displayed considerable antioxidant activities. In addition, compounds **5-7** exhibited potential antioxidant capacities in the ABTS assay.
Keywords – *Phlomis umbrosa*, triterpenoids, phenylethanoids, phenolics, antioxidant

Introduction

Several studies have indicated that free radicals and reactive oxygen species are the cause of several chronic diseases such as cancer, diabetes, atherosclerosis, and several other cardiovascular diseases.[1] The anti-oxidant substances can protect the human body against the harmful effects of free radicals and reactive oxygen species or can reduce the progression of many chronic diseases. The antioxidant capacity of compounds isolated from medicinal and food plants have received increasing attention due to the crucial role in disease prevention.[2]

Phlomis umbrosa Turcz., a species belongs to the Labiatae family, is distributed in several countries in Southeast Asia. In Korea, the dried roots of *P. umbrosa* are known as "Han Sok-Dan" and are used for treatment of hemorrhage, bronchitis, and cold.[3] Recently, *P. umbrosa* was known as increasing the longitudinal bone growth rate and was used for children with short stature.[3] Previous studies on the roots of *P. umbrosa* have shown that they possess diverse chemical constituents such as phenolics, iridoids, flavonoids, phenylpropanoids, lignans, neolignans, diterpenoids, alkaloids and essential oils.[3,4] Some of the important biological effects of this plant may be related to the presence of flavonoids and phenolic compounds. The chemical constituents from the roots of *P. umbrosa* have been reported to show multiple biological and pharmacological activities such as anti-nociceptive, anti-inflammatory, and anti-allergic effects.[3] The purpose of this study is the isolation of chemical constituents and the investigation of their antioxidant activities in the DPPH and ABTS assays.

Experimental

General experimental procedures – The specific rotations were operated on a JASCO DIP-370 digital Polarimeter. NMR spectra were performed in methanol-d_4 on an Oxford AS 400 MHz instrument (Varian, Palo Alto, CA, USA). Column chromatography was conducted using silica gel (Merck, Darmstadt, Germany), RP-18 (Merck, Darmstadt, Germany), and Sephadex LH-20 (Pharmacia Co. Ltd.). Thin layer chromatography (TLC) tests were performed on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). Fractions were monitored by TLC and spots were visualized by spraying with 10% H_2SO_4 in ethanol solution, followed by heating. The semi-preparative HPLC systems (Gilson, Middleton, WI, USA) were carried out with an ultraviolet (UV) detector (UV/VIS 151) and an Optima Pak C-18 column (10 × 250 mm, 10 μm particle

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size, RS Tech. Corp., Korea). All other agents and solvents were analytical grade.

Plant material – The roots of *P. umbrosa* were collected in Yeongwol-gun, Gangwon-do, Korea, in March 2016. The plant was identified and authorized by Professor Byung Sun Min. A voucher specimen (PU.2016005) has been deposited at the Herbarium of College of Pharmacy, Catholic University of Daegu, Korea.

Extraction and isolation – The dried roots of *P. umbrosa* (15.0 kg) were extracted with 60% EtOH (3 × 36 L) at 80 ºC for 4 h. After drying under reduced pressure, the concentrated extract (5.2 kg) was suspended in H_2O (5.0 L) and partitioned successively with *n*-hexane (12 L × 5, 1.20 kg), EtOAc (12 L × 12, 1.31 kg), *n*-BuOH (12 L × 3, 0.35 kg), and H_2O-soluble fractions. The *n*-BuOH fraction (0.35 kg) was subjected to silica gel column chromatography, using a gradient solvent system of CH_2Cl_2/MeOH/H_2O (100:0:0 to 1:2:0.1) to give 15 fractions (PU1–PU15) according to their TLC profiles. Fraction PU9 (4.3 g) was subjected to RP-C18 column chromatography, eluted with a gradient solvent system of ACN/H_2O (1:4 to 4:1) to give eight sub-fractions (PU9A–PU9H). Sub-fraction PU9B (420 mg) was chromatographed on RP-C18 column chromatography, eluted with ACN/H_2O, to give **3** (29.1 mg, w/w: 0.0002%), **4** (8.7 mg, w/w: 0.00006%), and **6** (11.4 mg, w/w: 0.00008%), respectively. Sub-fraction PU9F (307 mg) was subjected to RP-C18 column with elution solvent of acetone/H_2O (1:3), to afford compounds **1** (9.4 mg, w/w: 0.00006%) and **2** (7.5 mg, w/w: 0.00005%), separately. Sub-fraction PU9G (324.0 mg) was subjected to a semi-preparative HPLC [RS Tech Optima Pak C18 column (10 × 250 mm, 10 μm particle size)] eluted with a gradient solvent system of ACN/H_2O (15% to 30% ACN in H_2O containing 0.1% formic acid) for 60 min, flow rate 2 mL/min, UV detection at 254 nm, to afford compounds **8** (16.1 mg, t_R = 20.2 min, w/w: 0.0001%), **9** (6.7 mg, t_R = 40 min, w/w: 0.00004%), **10** (7.4 mg, t_R = 32 min, w/w: 0.00005%), and **11** (15.8 mg, t_R = 16.3 min, w/w: 0.0001%), respectively. Sub-fraction PU12 (3.1 g) was chromatographed on RP-C18 column, eluted with a gradient solvent system of MeOH/H_2O (1:3 to 2:1), to give six fractions (PU12A–PU12F). Sub-fraction PU12B (230 mg) was loaded to open column chromatography on RP-18 eluting with a solvent system of acetone/water (1:1.5) to get compound **12** (13.2 mg, w/w: 0.00009%). Sub-fraction PU12E (507 mg) was purified by a semi- preparative HPLC eluting with a solvent system of ACN/H_2O (4:96 to 15:85) for 50 min, flow rate 2 mL/min, UV detection at 254 nm, to yield **5** (17.2 mg, t_R = 39.0 min, w/w: 0.0001%) and **7** (12.5 mg, t_R = 44.3 min, w/w: 0.00008%).

Ajunolic acid (1) – Colorless needles ^{1}H NMR (CD_3OD, 400 MHz): δ_H 1.63 (H-1a), 1.39 (H-1b), 3.67 (1H, td, *J* = 4.4, 11.2 Hz, H-2), 3.48 (1H, d, *J* = 11.1 Hz, H-3), 1.96 (1H, t, *J* = 6.0 Hz, H-5), 1.75 (1H, t, *J* = 7, 8 Hz, H-6a), 1.31 (1H, m, H-6b), 1.56 (1H, m, H-7a), 1.26 (1H, m, H-7b), 1.42 (1H, m, H-9), 3.24 (1H, d, *J* = 11.0 Hz, H-11a), 3.33 (1H, d, *J* = 9.6 Hz, H-11b), 5.24 (1H, br s, H-12), 3.29 (1H, m, H-16a), 1.81 (1H, d, *J* = 10.0 Hz, H-16b), 2.83 (1H, dd, *J* = 3.4, 13.5 Hz, H-18), 1.67 (1H, d, *J* = 15.0 Hz, H-19a), 1.23 (1H, d, *J* = 15.0 Hz, H-19b), 1.89 (1H, t, *J* = 4.0 Hz, H-21a), 1.87 (1H, m, H-21b), 3.24 (1H, m, H-23a), 3.35 (1H, m, H-23b), 0.80 (3H, s, H-24), 0.68 (3H, s, H-25), 0.93 (3H, s, H-26), 1.16 (3H, s, H-27), 0.93 (3H, s, H-29), 0.89 (3H, s, H-30); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 47.6 (C-1), 69.7 (C-2), 78.1 (C-3), 43.0 (C-4), 49.0 (C-5), 19.1 (C-6), 33.3 (C-7), 40.5 (C-8), 48.9 (C-9), 39.0 (C-10), 24.0 (C-11), 123.4 (C-12), 145.4 (C-13), 42.7 (C-14), 28.8 (C-15), 24.6 (C-16), 47.2 (C-17), 44.1 (C-18), 48.9 (C-19), 31.8 (C-20), 33.8 (C-21), 33.6 (C-22), 66.2 (C-23), 13.9 (C-24), 17.8 (C-25), 19.1 (C-26), 26.5 (C-27), 181.8 (C-28), 24.6 (C-29), 24.0 (C-30).

Belleric acid (2) – Colorless needles ^{1}H NMR (CD_3OD, 400 MHz): δ_H 1.69 (H-1a), 1.38 (H-1b), 3.61 (1H, d, *J* = 11.4 Hz, H-2), 3.47 (1H, d, *J* = 10.6 Hz, H-3), 1.99 (1H, m, H-5), 1.74 (1H, m, H-6a), 1.36 (1H, m, H-6b), 1.55 (1H, m, H-7a), 1.24 (1H, m, H-7b), 1.38 (1H, m, H-9), 3.61 (1H, d, *J* = 11.4 Hz, H-11a), 3.33 (1H, d, *J* = 15.0 Hz, H-11b), 5.24 (1H, br s, H-12), 3.29 (1H, m, H-16a), 1.81 (1H, d, *J* = 9.6 Hz, H-16b), 2.84 (1H, d, *J* = 10.3 Hz, H-18), 1.69 (1H, m, H-19a), 1.24 (1H, m, H-19b), 1.74 (1H, m, H-21), 1.92 (1H, m, H-21), 3.33 (1H, m, H-23a), 3.43 (1H, d, *J* = 9.6 Hz, H-23b), 3.47 (1H, d, *J* = 10.7 Hz, H-24a), 3.82 (1H, m, H-24b), 0.78 (3H, s, H-25), 1.01 (3H, s, H-26), 1.16 (3H, s, H-27), 0.93 (3H, s, H-29), 0.90 (3H, s, H-30); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 47.7 (C-1), 69.8 (C-2), 79.4 (C-3), 47.6 (C-4), 48.2 (C-5), 19.6 (C-6), 34.9 (C-7), 40.5 (C-8), 49.1 (C-9), 38.8 (C-10), 24.8 (C-11), 123.3 (C-12), 145.4 (C-13), 42.7 (C-14), 28.8 (C-15), 24.0 (C-16), 47.6 (C-17), 42.9 (C-18), 47.2 (C-19), 31.6 (C-20), 33.6 (C-21), 31.6 (C-22), 64.5 (C-23), 62.6 (C-24), 17.6 (C-25), 17.4 (C-26), 26.4 (C-27), 181.8 (C-28), 33.8 (C-29), 24.0 (C-30).

Martynoside (3) – Pale powder, ^{1}H NMR (CD_3OD, 400 MHz): δ_H 6.73 (1H, s, H-2), 6.68 (1H, d, *J* = 8.2 Hz, H-5), 6.81 (1H, d, *J* = 8.2 Hz, H-6), 3.91 (1H, m, H-α), 3.71 (1H, m, H-α), 2.82 (2H, t, *J* = 6.9 Hz, H-β), 3.88 (3H, s, 4-OCH_3), 7.19 (1H, s, Hz, H-2'''), 6.73 (1H, d, *J* = 8.2 Hz, H-5'''), 7.08 (1H, d, *J* = 8.2 Hz, H-6'''), 6.37 (1H, d, *J* = 15.9 Hz, H-α'), 7.65 (1H, d, *J* = 15.9, H-β'),

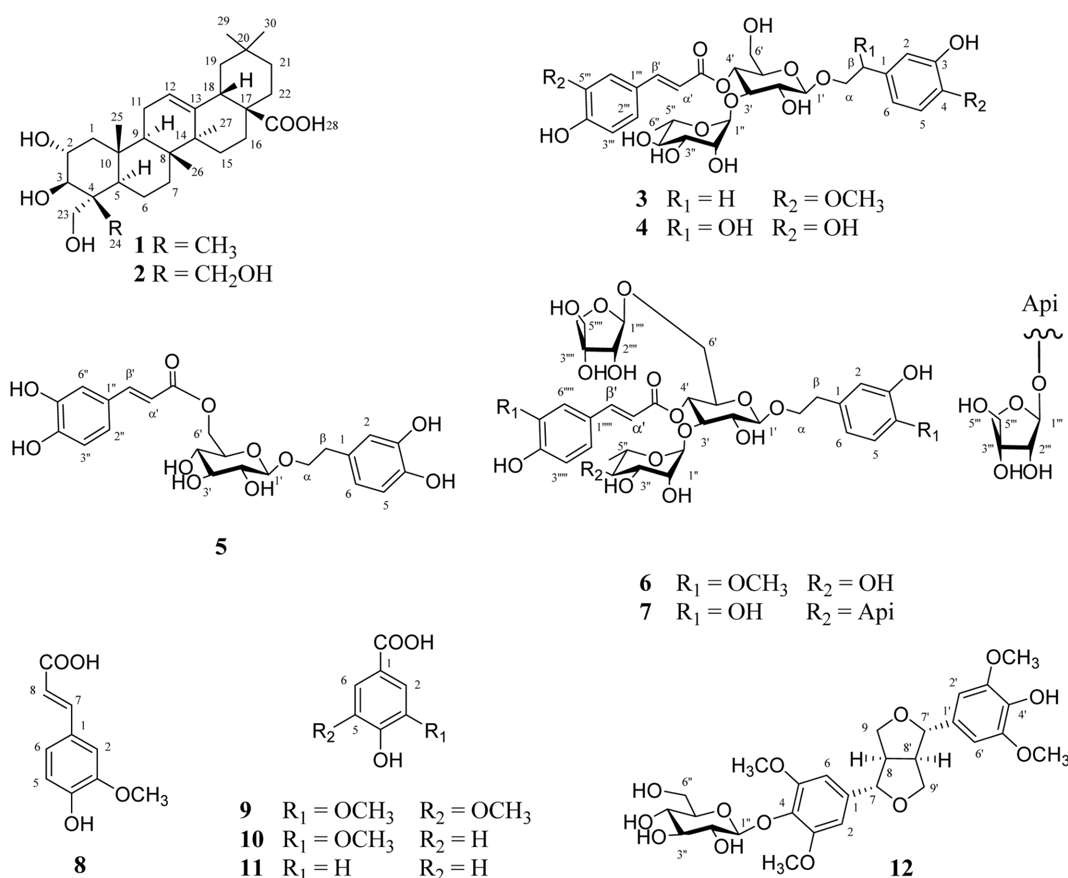

Fig. 1. The structures of isolated compounds (**1** - **12**) from the roots of *P. umbrosa*.

3.81 (3H, s, 5'''-OCH_3), 4.37 (1H, d, J = 7.9 Hz, H-1'), 3.56 (1H, m, H-2'), 3.81 (1H, m, H-3'), 4.92 (1H, m, H-4'), 3.67 (1H, m, H-5', 6'a), 3.38 (1H, m, H-6'b), 5.19 (1H, s, H-1''), 4.05 (1H, m, H-2''), 3.56 (1H, m, H-3''), 3.50 (1H, m, H-4''), 3.81 (1H, m, H-5''), 1.09 (3H, d, J = 6.2 Hz, H-6''); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 132.9 (C-1), 121.1 (C-2), 147.9 (C-3), 147.4 (C-4), 116.5 (C-5), 119.4 (C-6), 36.6 (C-β), 76.2 (C-α), 132.9 (C-1'''), 115.1 (C-2'''), 147.4 (C-3'''), 149.4 (C-4'''), 117.1 (C-5'''), 123.9 (C-6'''), 112.8 (C-α'), 147.5 (C-β'), 168.2 (COO), 104.2 (C-1'), 76.2 (C-2'), 81.5 (C-3'), 70.4 (C-4'), 73.8 (C-5'), 62.4 (C-6'), 103.0 (C-1''), 72.1 (C-2''), 72.1 (C-3''), 73.8 (C-4''), 70.4 (C-5''), 18.4 (C-6'').

Orobanchoside (4) – Pale powder, ^{1}H NMR (CD_3OD, 400 MHz): δ_H 6.78 (1H, s, H-2), 6.77 (1H, d, J = 6.4 Hz, H-5), 6.72 (1H, d, J = 6.4 Hz, H-6), 3.90 (1H, m, H-α), 3.72 (1H, m, H-α), 3.65 (1H, m, H-β), 7.05 (1H, d, J = 1.7 Hz, H-2'''), 6.84 (1H, d, J = 8.2 Hz, H-5'''), 6.78 (1H, dd, J = 1.7, 8.2 Hz, H-6'''), 6.27 (1H, d, J = 15.9 Hz, H-α'), 7.59 (1H, d, J = 15.9, H-β'), 4.41 (1H, d, J = 7.1 Hz, H-1'), 3.42 (1H, m, H-2'), 3.87 (1H, t, J = 2.2 Hz, H-3'), 4.92 (1H, m, H-4'), 3.73 (1H, m, H-5', 6'a), 3.46 (1H, m, H-6'b), 5.20 (1H, d, J = 3.2 Hz, H-1''), 3.92 (1H, m, H-2''), 3.56 (1H, d, J = 9.7 Hz, H-3''), 3.42 (1H, m, H-4''), 3.71 (1H, m, H-5''), 1.09 (3H, t, J = 6.2 Hz, H-6''); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 129.6 (C-1), 114.8 (C-2), 146.3

Table 1. The antioxidant capacities of isolated compounds (**1** - **12**) in DPPH and ABTS assay

Compound	IC_{50} of DPPH (μM)	IC_{50} of ABTS (μM)
1	> 100	> 100
2	> 100	> 100
3	89.2 ± 2.5	> 100
4	91.4 ± 3.6	> 100
5	14.1 ± 1.4	56.3 ± 2.5
6	74.6 ± 2.4	87.3 ± 2.7
7	62.7 ± 1.9	70.1 ± 3.5
8	> 100	> 100
9	> 100	> 100
10	> 100	> 100
11	> 100	> 100
12	> 100	> 100
L-Ascorbic acid	2.4 ± 0.2	2.8 ± 0.1

(C-3), 146.1 (C-4), 116.5 (C-5), 119.0 (C-6), 73.0 (C-α), 78.1 (C-β), 127.6 (C-1'''), 115.2 (C-2'''), 146.6 (C-3'''), 149.8 (C-4'''), 116.2 (C-5'''), 123.2 (C-6'''), 114.6 (C-α'), 148.1 (C-β'), 168.1 (COO), 104.7 (C-1'), 75.1 (C-2'), 80.6 (C-3'), 70.4 (C-4'), 73.7 (C-5'), 65.6 (C-6'), 103.0 (C-1''), 72.3 (C-2''), 72.0 (C-3''), 73.7 (C-4''), 70.7 (C-5''), 18.4 (C-6'').

3,4-Dihydroxyphenethylalcohol-6-*O*-caffeoyl-*β*-D-glucoside (5) – Pale powder, ^{1}H NMR (CD_3OD, 400 MHz): δ_H 6.73 (1H, s, H-2), 6.59 (1H, d, *J* = 7.9 Hz, H-5), 6.69 (1H, d, *J* = 7.9 Hz, H-6), 3.89 (1H, dd, *J* = 4.9, 11.2 Hz H-α), 3.27 (1H, m, H-α), 2.83 (2H, t, *J* = 7.2 Hz, H-β), 7.08 (1H, s, H-2''), 6.82 (1H, d, *J* = 8.0 Hz, H-5''), 6.94 (1H, d, *J* = 8.0 Hz, H-6''), 6.33 (1H, d, *J* = 15.8 Hz, H-α'), 7.61 (1H, d, *J* = 15.8, H-β'), 4.39 (1H, d, *J* = 7.9 Hz, H-1'), 4.55 (1H, d, *J* = 11.5 Hz, H-2'), 3.89 (1H, m, H-3'), 4.00 (1H, t, *J* = 8.7 Hz, H-4'), 3.75 (1H, m, H-5', 6'a), 3.57 (1H, m, H-6'b); ^{13}C NMR (CD_3OD, 100 MHz): δ_H 131.4 (C-1), 117.1 (C-2), 147.2 (C-3), 146.8 (C-4), 115.1 (C-5), 121.3 (C-6), 72.4 (C-α), 36.6 (C-β), 127.7 (C-1''), 114.9 (C-2''), 149.6 (C-3''), 146.1 (C-4''), 116.4 (C-5''), 123.1 (C-6''), 116.5 (C-α'), 147.2 (C-β'), 169.1 (COO), 104.5 (C-1'), 75.5 (C-2'), 77.9 (C-3'), 71.7 (C-4'), 75.1 (C-5'), 64.6 (C-6').

Leucosceptoside B (6) – Pale powder, ^{1}H NMR (CD_3OD, 400 MHz): δ_H 6.75 (1H, d, *J* = 2.0 Hz, H-2), 6.83 (1H, d, *J* = 8.2 Hz, H-5), 6.71 (1H, dd, *J* = 2.0, 8.2 Hz, H-6), 4.04 (1H, m, H-α), 3.75 (1H, m, H-α), 2.85 (2H, t, *J* = 6.2 Hz, H-β), 3.83 (3H, s, 4-OCH_3), 7.22 (1H, d, *J* = 1.8 Hz, H-2'''''), 6.84 (1H, d, *J* = 8.2 Hz, H-5'''''), 7.11 (1H, dd, *J* = 1.8, 8.2 Hz, H-6'''''), 3.91 (3H, s, 5'''''-OCH_3), 6.39 (1H, d, *J* = 15.9 Hz, H-α'), 7.68 (1H, d, *J* = 15.9, H-β'), 4.39 (1H, d, *J* = 7.9 Hz, H-1'), 3.38 (1H, m, H-2'), 3.88 (1H, t, *J* = 2.2 Hz, H-3'), 4.97 (1H, t, *J* = 9.7 Hz, H-4'), 3.75 (1H, m, H-5', 6'a), 3.49 (1H, dd, *J* = 1.8, 12.0 Hz, H-6'b), 5.21 (1H, d, *J* = 1.6 Hz, H-1''), 3.94 (1H, dd, *J* = 2.0, 3.0 Hz, H-2''), 3.58 (1H, dd, *J* = 3.1, 9.4 Hz, H-3''), 3.29 (1H, t, *J* = 9.6 Hz, H-4''), 3.70 (1H, m, H-5''), 1.11 (3H, d, *J* = 6.2 Hz, H-6''), 4.92 (1H, d, *J* = 2.2 Hz, H-1'''), 3.88 (1H, d, *J* = 2.3 Hz, H-2'''), 3.75 (1H, d, *J* = 9.6 Hz, H-4'''a), 3.94 (1H, d, *J* = 9.7 Hz, H-4'''b), 3.57 (2H, s, H-5'''); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 132.9 (C-1), 117.1 (C-2), 147.4 (C-3), 147.5 (C-4), 112.9 (C-5), 121.2 (C-6), 72.2 (C-α), 36.6 (C-β), 56.4 (4-OCH_3), 127.7 (C-1'''''), 111.8 (C-2'''''), 149.4 (C-3'''''), 150.8 (C-4'''''), 115.2 (C-5'''''), 124.4 (C-6'''''), 115.2 (C-α'), 147.9 (C-β'), 56.5 (5'''''-OCH_3), 168.1 (COO), 104.3 (C-1'), 76.2 (C-2'), 81.5 (C-3'), 70.4 (C-4'), 74.6 (C-5'), 68.5 (C-6'), 103.0 (C-1''), 72.4 (C-2''), 72.1 (C-3''), 73.8 (C-4''), 71.0 (C-5''), 18.4 (C-6''), 111.1 (C-1'''), 78.1 (C-2'''), 80.6 (C-3'''), 75.1 (C-4'''), 65.7 (C-5''').

Lunariifolioside (7) – Pale powder, ^{1}H NMR (CD_3OD, 400 MHz): δ_H 6.67 (1H, s, H-2), 6.66 (1H, d, *J* = 8.0 Hz, H-5), 6.55 (1H, d, *J* = 8.0 Hz, H-6), 4.00 (1H, m, H-α), 3.71 (1H, m, H-α), 2.78 (2H, t, *J* = 6.3 Hz, H-β), 7.04 (1H, s, H-2'''''), 6.76 (1H, d, *J* = 8.2 Hz, H-5'''''), 6.94 (1H, d, *J* = 8.2 Hz, H-6'''''), 6.26 (1H, d, *J* = 15.9 Hz, H-α'), 7.57 (1H, d, *J* = 15.9, H-β'), 4.36 (1H, d, *J* = 7.7 Hz, H-1'), 3.38 (1H, m, H-2'), 3.79 (1H, m, H-3'), 4.86 (1H, m, H-4'), 3.71 (1H, m, H-5', 6'a), 3.70 (1H, d, *J* = 8.9 Hz, H-6'b), 5.16 (1H, s, H-1''), 4.00 (1H, m, H-2''), 3.73 (1H, m, H-3''), 3.59 (1H, t, *J* = 8.5 Hz, H-4''), 3.54 (1H, m, H-5''), 1.07 (3H, d, *J* = 6.0 Hz, H-6''), 4.86 (1H, m, H-1'''), 3.77 (1H, m, H-2'''), 3.89 (1H, d, *J* = 9.6 Hz, H-4'''a), 3.77 (1H, d, *J* = 8.9 Hz, H-4'''b), 3.54 (2H, s, H-5'''), 5.16 (1H, s, H-1''''), 3.89 (1H, d, *J* = 3.96 Hz, H-2''''), 4.00 (1H, m, H-4''''a), 3.89 (1H, d, *J* = 9.6 Hz, H-4''''b), 3.59 (1H, br s, H-5''''); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 131.4 (C-1), 117.1 (C-2), 146.1 (C-3), 144.7 (C-4), 116.3 (C-5), 121.2 (C-6), 72.4 (C-α), 36.6 (C-β), 111.0 (C-1'''''), 115.2 (C-2'''''), 146.8 (C-3'''''), 149.8 (C-4'''''), 116.5 (C-5'''''), 123.2 (C-6'''''), 114.7 (C-α'), 148.0 (C-β'), 168.3 (COO), 104.2 (C-1'), 76.2 (C-2'), 81.7 (C-3'), 70.6 (C-4'), 74.6 (C-5'), 68.5 (C-6'), 103.0 (C-1''), 72.3 (C-2''), 72.0 (C-3''), 80.6 (C-4''), 70.4 (C-5''), 18.5 (C-6''), 111.0 (C-1'''), 78.1 (C-2'''), 80.6 (C-3'''), 75.1 (C-4'''), 65.6 (C-5'''), 114.7 (C-1''''), 78.1 (C-2''''), 80.6 (C-3''''), 75.1 (C-4''''), 65.6 (C-5'''').

Ferulic acid (8) – Pale powder, ^{1}H NMR (CD_3OD, 400 MHz): δ_H 7.16 (1H, d, *J* = 1.6 Hz, H-2), 6.77 (1H, d, *J* = 8.2 Hz, H-5), 7.05 (1H, dd, *J* = 1.6, 8.2 Hz, H-6), 7.56 (1H, d, *J* = 15.9 Hz, H-7), 6.28 (1H, d, *J* = 15.9 Hz, H-8); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 127.8 (C-1), 111.6 (C-2), 150.5 (C-3), 149.3 (C-4), 116.4 (C-5), 124.0 (C-6), 56.4 (3-OCH_3), 146.9 (C-7), 115.9 (C-8), 171.0 (COOH).

Syringic acid (9) – Pale powder, ^{1}H NMR (CD_3OD, 400 MHz): δ_H 7.30 (2H, s, H-2, 6), 3.86 (6H, s, 2 × OCH_3); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 131.0 (C-1), 107.1 (C-2, 6), 147.6 (C-3, 5), 140.4 (C-4), 55.3 (2 × OCH_3), 171.8 (COOH).

Vanillic acid (10) – White powder, ^{1}H NMR (CD_3OD, 400 MHz): δ_H 7.52 (1H, s, H-2), 7.51 (1H, d, *J* = 8.6 Hz, H-5), 6.80 (1H, d, *J* = 8.6 Hz, H-6), 3.86 (3H, s, OCH_3); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 115.8 (C-1), 125.2 (C-2), 148.6 (C-3), 152.6 (C-4), 113.7 (C-5), 115.8 (C-6), 56.4 (3-OCH_3), 168.5 (COOH).

4-Hydroxybenzoic acid (11) – White powder, ^{1}H NMR (CD_3OD, 400 MHz): δ_H 7.85 (2H, d, *J* = 7.9 Hz, H-2, 6), 6.78 (2H, d, *J* = 7.9 Hz, H-3, 5); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 124.7 (C-1), 133.0 (C-2, 6), 116.0 (C-3, 5), 163.3 (C-4), 169.7 (COOH).

(+)-Syringaresinol-*β*-D-glucoside (12) – White powder,

^{1}H NMR (CD_3OD, 400 MHz): $[\alpha]_D^{25}$ –26.7° (*c* 1.0, MeOH); δ_H 6.71 (2H, s, H-2, 6), 4.76 (1H, d, J = 3.7 Hz, H-7), 3.12 (1H, m, H-8), 3.90 (1H, m, H-9a), 4.27 (1H, m, H-9b), 6.65 (2H, s, H-2', H-6'), 4.71 (1H, d, J = 4.2 Hz, H-7'), 3.14 (1H, m, H-8'), 3.90 (1H, m, H-9'a), 4.27 (1H, m, H-9'b), 3.85 (6H, s, 2 × OCH_3), 3.84 (6H, s, 2 × OCH_3), 4.85 (1H, d, J = 7.5 Hz, H-1''), 3.49 (1H, m, H-2''), 3.42 (1H, m, H-3'', 4''), 3.20 (1H, m, H-5''), 3.77 (1H, m, H-6''a), 3.67 (1H, dd, J = 5.2, 12.0 Hz, H-6'b); ^{13}C NMR (CD_3OD, 100 MHz): δ_C 139.5 (C-1), 104.8 (C-2), 154.4 (C-3, 5), 135.5 (C-4), 104.4 (C-6), 87.5 (C-7), 55.7 (C-8), 72.9 (C-9), 133.0 (C-1'), 104.8 (C-2'), 149.3 (C-3', 5'), 139.5 (C-4'), 104.8 (C-6'), 87.1 (C-7'), 55.7 (C-8'), 72.8 (C-9'), 57.1 (2 × OCH_3), 56.8 (2 × OCH_3), 105.3 (C-1''), 75.7 (C-2''), 77.8 (C-3''), 71.2 (C-4''), 78.3 (C-5''), 62.5 (C-6'').

Radical scavenging assay (DPPH) – The DPPH assay was performed to determine the free radical scavenging activity of isolated compounds. Briefly, a 0.20 mM solution of DPPH radical was previously prepared in methanol, after that 150 µL of this solution was mixed to 50 µL of each compounds at various concentrations (100, 50, 25, 1 µg/mL) in methanol. After 15 min incubation in the dark room, the decrease in the absorbance of the solution was measured on a Titertek microplate reader at 517 nm (Multiskan MCC/340, MKII Microplate Reader). L-Ascorbic acid was used as the positive control. DPPH radical scavenging activity was calculated as $[1 - (A_{sample} - A_{blank}) / A_{control}] \times 100$, where A_{sample} is the absorbance of DPPH solution (150 µL) with the sample solution (50 µL); $A_{control}$ is the absorbance of the DPPH solution (150 µL) with methanol (50 µL); A_{blank} is the absorbance of methanol (150 µL) with the sample solution (50 µL). The results were expressed as IC_{50} (50% inhibitory concentration) value.

Radical scavenging assay (ABTS) – A solution of cation-radical $ABTS^{\bullet+}$ was prepared using the reaction mixture of 5 mL of aqueous solution of ABTS (CAS 30931-67-0, Sigma-Aldrich, Saint, Louis, USA) at concentration of 7 mM and 88 µL of 140 mM $K_2S_2O_8$. The final working solution of $ABTS^{\bullet+}$ was obtained after reaction time from 12 to 16 h under the laboratory temperature at darkness. The ABTS reagent was diluted with acetate buffer (0.1 M, pH 5) to achieve the absorbance of 0.700 at 734 nm. For the spectrophotometric measurement of the samples, 1 mL of diluted $ABTS^{\bullet+}$ and 100 µL of sample were mixed. L-Ascorbic acid was used as the positive control. Calibration curve was prepared using L-Ascorbic acid at the concentration range of 0.5 - 5 µM. ABTS radical scavenging activity was calculated as $[1 - (A_{sample} - A_{blank}) / A_{control}] \times 100$, where A_{sample} is the absorbance of ABTS solution (150 µL) with the sample solution (50 µL); $A_{control}$ is the absorbance of the ABTS solution (150 µL) with methanol (50 µL); A_{blank} is the absorbance of methanol (150 µL) with the sample solution (50 µL). The results were expressed as IC_{50} (50% inhibitory concentration) value.

Result and Discussion

Based on the results of the present study, repeated chromatography of *n*-BuOH fraction from *P. umbrosa* led to the isolation of twelve compounds (**1 - 12**). Their chemical structures were determined by extensive analysis of the physicochemical and spectroscopic data, as well as by comparing with reported literatures. The isolated compounds (**1 - 12**) were identified as arjunolic acid (**1**),[5] belleric acid (**2**),[5] martynoside (**3**),[6] orobanchoside (**4**),[7] 3,4-dihydroxyphenethylalcohol-6-*O*-caffeoyl-*β*-D-glucoside (**5**),[8] leucosceptoside B (**6**),[9] lunariifolioside (**7**),[10] ferulic acid (**8**),[11] syringic acid (**9**),[12] vanillic acid (**10**),[13] 4-hydroxybenzoic acid (**11**),[5] and (+)-syringaresinol-*β*-D-glucoside (**12**).[14] To the best of our knowledge, compounds **3 - 9** and **12** were isolated for the first time from this plant.

The DPPH radical scavenging assay is the most frequently used method and it offers the first approach for evaluating the antioxidant capacity, since the DPPH assay is a valid, accurate, easy, and economical method. DPPH is a stable chromogen radical with a deep purple color. The DPPH scavenging assay is based on electron donation by antioxidants to neutralize the DPPH radical. The reaction is accompanied by color change of the DPPH measured at 517 nm, and the discoloration serves as an indicator of the antioxidant efficacy.

The ABTS assay measures the ability of antioxidants to scavenge the stable radical cation $ABTS^{\bullet+}$, a blue-green chromophore with maximum absorption at 734 nm which decreases in its intensity in the presence of antioxidants. $ABTS^{\bullet+}$ can be generated from ABTS in the presence of strong oxidizing agents. Antioxidants can neutralize the radical cation $ABTS^{\bullet+}$ by either direct reduction *via* electron donation or by radical quenching *via* hydrogen atom donation.

Compounds **1 - 12** were explored for their antioxidant potential *via* the DPPH and ABTS assays at various concentrations (1, 25, 50, and 100 µM). As a result, vitamin C, a commercial drug, was used as the positive control with IC_{50} values of 2.4 ± 0.2 and 2.8 ± 0.1 µM in the DPPH and ABTS assays, respectively. Compound **5**, a

phenylethanoid, showed potent antioxidant capacity (IC_{50} value of 14.1 ± 1.4 μM) in the DPPH assay. Other phenylethanoids, **6** and **7**, exhibited considerable antioxidant capacities with IC_{50} values of 74.6 ± 2.4 and 62.7 ± 1.9 μM, respectively. Compounds **3** and **4** displayed lower antioxidant capacities with IC_{50} values of 89.2 ± 2.5 and 91.4 ± 3.6 μM, respectively. Thus, these phenylethanoid glucosides in this plant possess potential antioxidant activity. This may be correlated to the number of free hydroxyl groups in their structures that served as hydrogen donors. In contrast, two triterpenoids (**1** and **2**), four phenolics **(8 - 11)**, and one lignan (**12**), showed weak or inactive antioxidant capacity due to IC_{50} values > 100 μM in the DPPH assay. In the ABTS assay, compounds **5 - 7** also displayed considerable antioxidant capacities with IC_{50} values of 56.3 ± 2.5, 87.3 ± 2.7, and 70.1 ± 3.5 μM, respectively. Numerous hydroxyl groups in the structures of these compounds may neutralize the radical cation ABTS by either direct reduction *via* electron donation or by radical quenching *via* hydrogen atom donation. Meanwhile, compounds **(1 - 4** and **8 - 12)** showed no activity (IC_{50} values > 100 μM). All phenylethanoid compounds (**3 - 7**) isolated from roots of *P. umbrosa* showed significant antioxidant activities in the DPPH or ABTS assay. Compounds **4**, **5**, and **7** were evaluated for their antioxidant capacity in the DPPH and ABTS assays for the first time.

Arjunolic acid (**1**) is an oleanane-type triterpenoid. It is well known as a phytomedicine with multifunctional therapeutic applications such as prevention of myocardial necrosis, platelet aggregation, and coagulation. It possesses anti-inflammatory, antidiabetic, antitumor, and antimicrobial activities.[15] It has previously been reported that martynoside (**3**) possesses multiple biological activities such as antibacterial, antifungal, anticancer, and antimetastatic properties.[16,17] Lunariifolioside (**7**) is known as an α-glucosidase inhibitor.[18] A lignan isolated from this plant was reported to suppress proinflammatory mediators in SW982 human synovial sarcoma cells.[19] Ferulic acid (**8**) is abundant in many plants such as rice, wheat, oats, grasses, grains, vegetables, flowers, and fruits. Ferulic acid has been shown to have antidiabetic, anti-ageing, and anticancer activities.[20] Syringic acid (**9**) is found in many plants and food. Various biological activities of syringic acid like antiproliferative, anti-endotoxic, and anti-cancer activities have been reported.[21] Vanillic acid (**10**) is a flavoring agent found in edible plants and fruits. It has been previously reported to possess anti-nociceptive, anti-inflammatory, and antibacterial activities.[22] Another phenolic acid, 4-hydroxybenzoic acid (**11**), is known to have antimicrobial activity. It plays a role in protecting plants from pathogenic microorganisms.[23]

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